multicistronic transgenes, and should be considered alongside other factors to guide the assembly of an optimal vectorized monoclonal antibody expression cassette.

275. Development of Universal, Strong Mini-Promoters for Recombinant Adeno-Associated Viral (rAAV) Vectors

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rAAVs have emerged as an efficient gene delivery tool. Discovery of various natural serotypes and recent development of recombinant capsids significantly advanced the transduction efficiency of rAAVs in a variety of cells and tissues. On the other hand, much less effort has been made for maximizing expression of the rAAV cargo DNA, since current AAV vectors mainly rely on well-established promoters for gene expression. Among those, CMV and CAG promoters belong to the most frequently used strong promoters providing universal activity. The capacity of DNA packaging in rAAV capsids is limited (4.7 kb). Hence, the large size of the existing strong promoters is a drawback in delivering genes and gene editing tools of large sizes, reaching the limits of the viral packaging capacity. To improve AAV as a gene therapy tool, discovery of small but strong promoters is a crucial step. Here we report two new strong mini promoters, called INS84 and GCG110, with universal activity in rAAV expression vectors. These promoters are only 84 and 135 base pairs in size, respectively. They showed strong expression of a reporter transgene from rAAV in human and mouse cells and tissues, including human hepatocytes in primary cultures, humanized mice in vivo and human pancreatic islet cells. Expression levels in these tissues were comparable to those obtained with the much larger CAG promoter. Until now, viral vectors utilized (or 'borrowed') promoters that are characterized in the context of plasmid expression vectors or germ-line transgenes. Our strong mini-promoters for rAAV expression suggest a new direction for developing promoters for viral vectors, specifically that the large size of promoters required for expression in the context of plasmid vectors is often not necessary for strong expression in an rAAV vector.

276. Deep Tropism Profiling of Barcoded AAV Capsid and Cargo Pools in Intact Tissue Using High-Throughput Ultrasensitive Sequential FISH

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Genetic access to specific cell types through minimally invasive routes is of particular interest in basic research and clinical applications. Extensive efforts have been made in engineering gene delivery vectors, such as recombinant adeno-associated viruses (rAAVs), and gene regulatory elements to achieve this goal. Despite many interesting candidates, revealed for example from directed evolution via M-CREATE (Sripriya Ravindra Kumar *et al.*, *Nature Methods*, 2020), histology-based characterization presents a bottleneck due to the limited number of variants and/or cell types that can be investigated at once. To address this, we have developed ultrasensitive sequential FISH (useqFISH) for multiplexed detection of both endogenous and barcoded transgene transcripts in intact tissue with single-molecule resolution. By combining two amplification strategies (rolling circle amplification, RCA, and hybridization chain reaction, HCR), we achieved a 2.7- or 6.7-fold increased signal-to-background ratio of useqFISH in comparison to one with RCA or HCR only amplification, respectively. UseqFISH allowed us to detect endogenous genes with a single probe pair (20-nucleotide (nt) for each) and, in transfected cell cultures, to distinguish capsid variants with genomes differing by only 7-mer peptide modification. We further improved useqFISH by establishing an automated single-molecule imaging and microfluidic solution exchange system and an analytical pipeline for 3D imaging data. To demonstrate the applicability of useqFISH for in vivo AAV profiling, we employed this method to further characterize a pool of 6 AAV capsid variants that we found to be highly efficient for brain-wide and/or cell-type biased transduction in the mouse brain following systemic delivery. We designed unique nucleic acid barcodes (160-nt) in the 3'UTR of each viral genome and retro-orbitally injected the pooled AAVs into 2 C57BL6/J mice at a dose of 5e10 viral genomes (vg) per variant (total 3e11 vg/mouse). For transcript detection, 11 canonical cell-type markers (e.g., Slc17a7, Gad1, Pvalb, SST, VIP, etc) were used together with probes against the viral genome barcodes, to characterize the cell-type tropisms of each variant. Next, we designed a pool of 103 barcoded AAV genomes carrying 4 tandem repeats of a unique miRNA target site. We packaged these genomes into AAV-PHP.eB and delivered to 3 C57BL6/J mice at a dose of 1e10 vg/variant (total ~1e12 vg/mouse). Using useqFISH, we were able to assess the ability of each miRNA target site to dampen transgene expression in different cell types, thereby revealing useful intersectional strategies to refine cell-type-specific transgene expression with capsid/cargo combinations. These results demonstrate that useqFISH allows for high-throughput characterization of pooled genetic variants of viral capsids and gene regulatory elements in intact tissue and thus enables comprehensive profiling of genetic toolkits for precise access to targets of interest.

277. Simultaneous Detection of AAV Genome, Transcript, and Protein Localization in Intact Cells and Tissues at High Resolution

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Recent years have seen significant progress in AAV capsid engineering for gene delivery with increased efficiency and desired cell-type specificity to match the needs of pre-clinical research and gene therapy. The cellular uptake of AAVs, however, can stop short of the ultimate goal of cargo protein production - due to AAV silencing, insufficient nuclear transport, inefficient uncoating, failed second-strand synthesis, or other still to be discovered mechanisms. Defining the relationship between AAV genome uptake, transcription and cargo protein synthesis efficiencies in different cell types and tissues can help bypass key bottlenecks in gene delivery and guide effective AAV engineering. We adapted a recently published in-situ-transcription-based signal amplification method, the "Zombie technique" (*1*), to detect AAV genomes in a variety of fixed cells and tissues. Zombie involves producing 20 to 380 base-pair long barcode transcripts by exogenously-supplied T7 phage polymerase, which are then visualized at high

resolution in intact cells through fluorescent in situ hybridization (FISH) by hybridization chain reaction (HCR). We demonstrate that Zombie enables simultaneous detection of AAV genomes and AAV transcripts by FISH and cargo proteins by immunofluorescence. Consequently, we can determine the rates of AAV genome nuclear uptake, cargo transcription and cargo translation at different time points post-infection. Combining the Zombie method with cell-type-specific markers (protein immunolabelling or mRNA FISH) can give insights into subcellular AAV processing in complex tissues and help pinpoint to limiting factors in the overall transduction process. Interestingly, we find that after direct brain injection of AAV6, although cargo genomes do enter the nuclei of microglia, these genomes do not result in functional cargo protein production in this cell type. Furthermore, we analyze the pattern of AAV genome nuclear uptake, transcription and translation in mice after systemic delivery of AAV9 and engineered AAV capsids (AAV.PHP.eB, AAV.CAP-B10) (*2, 3*) across brain, liver and spleen. In conclusion, the Zombie technique adapted to AAV vectors enables simultaneous cargo genome, mRNA and protein visualization and thus can help differentiate cellular and subcellular processing steps of engineered gene delivery vectors, facilitating their refinement for basic and translational research.

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2. K. Y. Chan *et al.*, *Nat. Neurosci.* **20**, 1172-1179 (2017).
3. N. Flytzanis *et al.*, bioRxiv doi.org/10.1101/2020.06.16.152975 (2020).

278. Building a Stable Transgene Expression Cassette Plasmid to Enhance the Quality and Potency of AAV Gene Therapy Vectors

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The use of adeno-associated virus (AAV) for gene therapy has been accelerated in recent years. Large-scale AAV vector production with consistent purity and potency remains a challenging hurdle to overcome. DNA deletions and mutations within the inverted terminal repeats (ITR) of the transgene plasmid can occur during bacterial plasmid amplification and AAV production processes thereby hampering vector quality. Here we describe engineered ITR transgene plasmids containing both stable and unstable ITR sequences and further evaluated AAV packaging quality and potency. The occurrence of ITR mutations and deletions within the stable ITR plasmid was confirmed to be significantly reduced during large-scale plasmid production and purification. Packaged ssDNA content in AAV vectors that were generated using various ITR plasmids was also examined by next-generation sequencing to identify transgene sequences and non-transgene DNA fragments. Moreover, we evaluated AAV vector potency in vitro and in vivo by measuring the expression and activity of a reporter gene under the control of various regulatory elements. Our results indicate that AAV vector production and potency benefits from the use of a stable ITR transgene plasmids.

279. Abstract Withdrawn

280. Abstract Withdrawn

281. Rational Design of Short Constitutive Promoters for Improved Transgene Expression in AAV Vectors

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Recombinant AAV vectors are emerging as powerful delivery vehicles for human gene therapy, with three approved AAV gene therapy products to date and many more in clinical trials. In order to maximize the utility of AAV vector- mediated gene delivery, it is necessary to design the next generation AAV vectors with improved characteristics. Considerable attention has been focused on capsid engineering, however, of equal importance is the optimization of regulatory components of the AAV vector genome itself. In particular, novel constitutive synthetic promoters are needed that can be easily packaged into AAV vectors to improve transgene expression characteristics including cell-type specificity, prevent toxicity from overexpression and reduce innate immune responses to the promoter itself. Here, we describe a rational promoter engineering approach to generate short (300-700 bp) synthetic promoters for constitutive transgene expression from AAV vectors with enhanced expression profiles. Core promoters were subject to modification of key regulatory sites including the initiator site, TATA box, GC box and DPE site. In addition, various modified introns were inserted into the 5' UTR region of these core promoters, and enhancer elements were added upstream of the modified core promoters. All candidates tested were first evaluated *in vitro* in HEK293T cells, using a dual luciferase expression system and compared to the commonly used strong tCAG promoter and the weak EFS promoter. Novel synthetic promoters that were between 300-700 bp in size, demonstrated increased expression compared to EFS, and showed a larger dynamic range of expression with less saturation compared to EFS and tCAG were then selected for testing in vivo. AAV vectors with candidate engineered synthetic promoters driving fluorescent reporter transgenes were packaged into different serotypes and delivered to the CNS and muscle of mice. From this study we identified novel short synthetic promoters that demonstrated improved transgene expression characteristics compared to existing constitutive promoters both in vitro and in vivo.